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Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (*Argyrosomus regius*)

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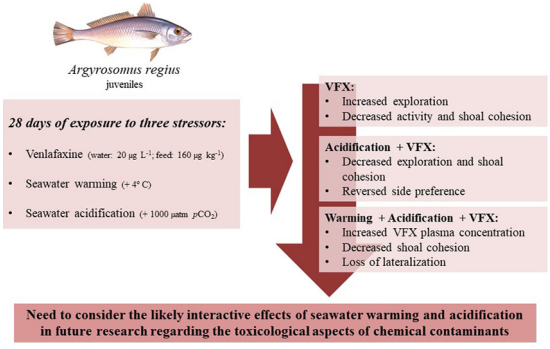
HIGHLIGHTS

- Warming and acidification enhanced VFX bioaccumulation in fish plasma.
- VFX triggered fish exploration, but reduced fish activity and shoal cohesion.
- Altered temperature and pH reduced shoal cohesion regardless of VFX exposure.
- Acidification plus VFX exposure reduced fish side preference.

GRAPHICAL ABSTRACT



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ABSTRACT

Antidepressants, such as venlafaxine (VFX), which are considered emerging environmental pollutants, are increasingly more present in the marine environment, and recent evidence suggest that they might have adverse effects on fish behaviour. Furthermore, altered environmental conditions associated to climate change (e.g. warming and acidification) can also have a determinant role on fish behaviour, fitness and survival. Yet, the underlying interactions between these environmental stressors (pharmaceuticals exposure and climate change) are still far from being fully understood. The aim of this study was to assess behavioural responses (in juvenile meagre (*Argyrosomus regius*) exposed to VFX via water ([VFX] ~20 µg L^{-1}) and via dietary sources ([VFX] ~160 µg kg^{-1} dry weight), as well as to increased temperature ($\Delta T°C = +5$ °C) and high CO_2 levels (ΔpCO_2 ~1000 µatm; equivalent to $\Delta pH = -0.4$ units). Overall, VFX bioaccumulation in fish plasma was enhanced under the combination of warming and acidification. VFX triggered fish exploration, whereas fish activity and shoal cohesion were reduced. Acidification alone decreased fish exploration and shoal cohesion, and reversed fish preference to turn leftwards compared to control conditions. Such alterations were further enhanced by VFX exposure. The combination of warming and acidification also reduced shoal cohesion and loss of lateralization,

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regardless of VFX exposure. The distinct behaviour observed when VFX contamination, acidification and warming acted alone or in combination highlighted the need to consider the likely interactive effects of seawater warming and acidification in future research regarding the toxicological aspects of chemical contaminants.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) have become a great environmental concern, since they are a group of compounds intensively and continuously used, and their presence in both the environment and biota is currently not regulated. Domestic, hospital and industrial effluents, agriculture and aquaculture activities are the main sources of PPCPs' contamination in marine environments (Gros et al., 2012), and their elimination through conventional wastewater treatments is chemically dependent and only partial (e.g. 50% of maximum removal for venlafaxine, lidocaine and tramadol; Rúa-Gómez and Püttmann, 2012). Given their ability to easily cross biological membranes, and high specificity and effectiveness to target cells and tissues, PPCPs can be toxic to non-target organisms, even at very low concentrations (e.g. Mehinto et al., 2010; Schmidt et al., 2011). Such impacts may be more deleterious when long-term or chronic exposure occurs, particularly in early life stages that are known to possess lower capabilities to metabolize such contaminants (Richardson and Ternes, 2011). Nevertheless, the ecological impacts of PPCP exposure still require a better understanding, as most available studies do not consider chronic (but sublethal) exposures (Dulawa et al., 2004), and are focused on bioconcentration rates (i.e. contaminants exposure via water), while other exposure routes, such as trophic transfer (i.e. dietary exposure) have deserved very little attention, despite they can be particularly important in predatory fish species that have long life cycles and are able to reach relatively high body dimensions (Dijkstra et al., 2013; Brooks, 2014; Zenker et al., 2014; Maulvault et al., 2016).

Furthermore, very few studies consider the effect of other stressors, such as climate change (e.g. warming, acidification), which can affect PPCPs' bioavailability (Brooks, 2014).

Within pharmaceuticals of human use, venlafaxine (VFX) is frequently detected in the aquatic environment (e.g. around 50 ng L^{-1} in seawater samples and up to 580 ng L^{-1} in wastewater influent; Gros et al., 2012), often reaching higher concentrations than other well-known psychiatric drugs, such as fluoxetine or carbamazepine (Gros et al., 2012; Fong and Ford, 2014). VFX acts as a behaviour modulator by blocking the presynaptic reuptake of serotonin and norepinephrine (Serotonin-norepinephrine reuptake inhibitor; SNRI). This results in increased serotonin and norephinephrine in the synapse, which then is available to bind to postsynaptic receptors and cause increased downstream effects (Thaler et al., 2012). From the evolutionary perspective, vertebrate species have many preserved neurotransmitter systems and receptors, which is why many antidepressants that act on humans have similar effects on fish (e.g. Valenti et al., 2012; Bisesi Jr et al., 2014). However, studies on antidepressants and fish are limited and detailed toxicological information is required to better understand the effects of these compounds as wastewater pollutants (Brodin et al., 2013; Hamilton et al., 2017). Empirical data establishing toxicological and behavioural similarities (or distinctions) between humans, primates and other vertebrate organisms, such as fish, exposed to antidepressants are of important in two ways: i) to investigate fish species' potential as in vivo experimental models that complement the data provided by mammalian models in neurotoxicological studies, since laboratory studies using humans and primates are often difficult, time consuming, costly and underlie many ethical issues; and ii) to assess possible ecological implications and cascading effects to marine biota due to the environmental contamination related to human pharmaceuticals. Over the last decades, different tests have been developed and validated to assess distinct behavioural cues in fish species, such as animal anxiety (e.g. novel tank diving test; Bencan et al., 2009; Sackerman et al., 2010; Reyhanian et al., 2011; Stewart et al., 2012) and social interactions (e.g. the shoaling test; Moretz et al., 2007; Reyhanian et al., 2011). Moreover, during the last two decades, fish lateralization has been one of the main research areas in fish behavioural studies (e.g. Bisazza and Brown, 2011; Bibost and Brown, 2013; Sampaio et al., 2016), because: i) it is intrinsically involved in habitat exploration, synchronized and polarized group swimming (schooling), as well as in fish loose group aggregation (shoaling), thus contributing to enhance foraging and predator escape (e.g. Bisazza and Dadda, 2005; Bibost and Brown, 2013); ii) recent evidence suggest that lateralization is an ecological strategy required to meet the contemporary ecological and social demands involved in the processes of natural selection (e.g. Bisazza and Dadda, 2005; Bisazza et al., 2000; Bisazza and Brown, 2011; Bibost and Brown, 2013).

One third of the anthropogenically-originated carbon dioxide (CO_2) has been absorbed by the oceans, which has led to a 0.1 unit drop in seawater pH from the pre-industrial to the present days (IPCC, 2014). Carbon dioxide concentrations have risen to concentrations now exceeding 400 ppm (NOAA, 2017), and are expected to reach ~900 ppm by the end of the 21st century (Pörtner et al., 2014). These consequent changes in seawater chemistry are underpinned by a net increase of hydrogen (H^+) and bicarbonate (HCO_3^-) ions and decrease in carbonate ions (CO_3^{2-}), a process known as ocean acidification (Caldeira and Wickett, 2003). By 2100, in a "business-as-usual" scenario, the continuous CO_2 uptake is expected to elicit a further 0.13–0.42 pH drop (IPCC, 2014). Concomitantly, excessive greenhouse gas emissions (which are responsible for heat absorption and reemission) are also expected to promote a surface seawater temperature increase as high as +4.8 °C (IPCC, 2014). Given the susceptibility of marine organisms to environmental variations, which can affect their physiological status and behaviour (e.g. Anacleto et al., 2014; Sampaio et al., 2016; Rosa et al., 2017), warming and acidification are two of the main challenges that species will have to face in a changing ocean (IPCC, 2014). By interfering with seawater physical and chemical properties, climate change can also affect the availability of chemical contaminants in marine ecosystems, their transfer among environmental compartments and their toxicity to biota (Marques et al., 2010; IPCC, 2014). Yet, it is still unclear how species will cope with the presence of chemical contaminants in climate change scenarios. Since behaviour plays a major role in an organism's ecological fitness and survival, potential changes induced by chemical contaminants and climate change, as well as the combination of both stressors may lead to substantial consequences at populational and ecosystem levels.

Within this context, the present study aimed to assess VFX bioaccumulation (fish plasma) and the respective behavioural responses (anxiety, swimming activity, shoaling and lateralization) in juvenile meagre (*Argyrosomus regius*), when accounting for the effects of: a) VFX exposure route (via water, i.e. [VFX] ~20 μg L^{-1}, and via dietary sources, i.e. [VFX] ~160 μg kg^{-1}, dw); b) abiotic stressors, namely warming ($\Delta T°C = +5\,°C$) and acidification (ΔpCO_2 ~1000 μatm; $\Delta pH = -0.4$ units). Juvenile *A. regius* was selected as biological model because it is a predatory fish species that typically inhabits estuaries and coastal areas, thus being susceptible to accumulate high levels of chemical contaminants (FAO, 2017). Furthermore, the fact that it is also a commercially valuable species also emphasizes the relevance of using this species in ecotoxicological and behavioural studies, as changes to the behavioural patterns can

potentially affect juvenile recruitment and species ecological success, therefore, affecting both fisheries and aquaculture sectors in an adverse way.

2. Materials and methods

2.1. Feeds (CTR and VFX-enriched) and VFX stock solutions

Non-contaminated feed (control, CTR feed) and VFX contaminated feed (VFX-enriched feed) with the same nutritional composition were manufactured by the company SPAROS Lda (Olhão, Portugal). Detailed feed composition can be consulted in *Supplementary Materials* Table S1. Briefly, a control diet (CTR feed) was formulated to mimic a commercial fishmeal-rich formulation for juvenile marine fish with 48% crude protein and 18% crude fat. All powder ingredients were grinded (<200 μm) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Ingredients and fish oil were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain), and the feed mixture was further humidified with 25% deionized water at room temperature. The diet was extruded at 2.0 mm by means of a low-shear extruder (P55, Italplast, Italy). Upon extrusion, the feed pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). A 10 kg batch of CTR feed was subsequently contaminated with VFX (VFX-enriched feed). Given the current lack of background information, and to assure that behavioural changes were elicited during the timeline of the trials, a VFX nominal concentration of approximately 160 μg kg^{-1} on a dry weight basis (dw) was selected, which corresponds to ~4 times the values commonly found in species inhabiting contaminated coastal areas, susceptible to accumulate this contaminant, and that are natural preys of juvenile meagre (Álvarez-Muñoz et al., 2015). To prepare the VFX-enriched feed, venlafaxine hydrochloride ($C_{17}H_{27}NO_2 \cdot HCl$, >98%, CAS Number 99300-78-4, Sigma-Aldrich) previously solubilized in ethanol, was further diluted in deionized water (total volume of 100 mL), and this solution was top-coated to the pellets with a pressurized spraying container (standard flat-fan nozzle; size 250 μm; pressure 6 bar). Despite the top-coating process followed leads to the total volatilization of organic solvents (and, therefore, ethanol is not expected to be present in the experimental feeds), equivalent amounts of ethanol were also added to the Control feed to rule out the possibility of occurring any solvent carrier toxicity through feed.

To perform VFX exposure via water (i.e. in VFX-water treatment), a stock solution of VFX was prepared to daily spike seawater during the 28 days of exposure, by dissolving venlafaxine hydrochloride ($C_{17}H_{27}NO_2 \cdot HCl$, >98%, CAS Number 99300-78-4, Sigma-Aldrich) with deionized water (total volume of 500 mL), in order to achieve a nominal VFX concentration of 20 μg L^{-1} in each incubating tank. Such VFX nominal concentration was mostly based on the order of magnitude of the lowest VFX concentration previously reported to cause significant behavioural effects in fish following short-term VFX exposure (50 μg L^{-1}; Bisesi Jr et al., 2014).

2.2. Fish rearing and acclimation

A. regius specimens ($n = 135$) with similar biometric characteristics were reared until juvenile stage (total length: 6.8 ± 0.6 cm; weight 2.6 ± 0.8 g; Table 1) at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) using routine hatchery conditions. Subsequently, fish were transported to the aquatic facilities of Laboratório Maritimo da Guia (MARE-FCUL, Cascais, Portugal), where they were randomly and equitably distributed in 27 rectangular shaped incubating glass tanks (3 replicates × 9 treatments = 27 tanks in total; treatments randomly assigned to each tank/replicate; Fig. 1; see the description of each treatment in Sections 2.3.1 and 2.3.2), within independent recirculation aquaculture systems (RAS), each having 50 L of total volume capacity. Each of the 27 tanks had independent functioning, being equipped with protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal) to maintain seawater quality. Furthermore, each tank had independent temperature and pH control, and these parameters were adjusted whenever needed by means of: i) temperature - an automatic seawater refrigeration system (±0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as submerged digital thermostats (200 W, V2Therm, TMCIberia, Portugal); ii) pH - individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; scale: pH 0.0–14.0 units; Profilux 3.1N, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted whenever need, via submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or by CO_2-filtered aeration (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd., United Kingdom). Dead fish and faeces were daily removed and 25% seawater renewal was performed in each incubation tank. Ammonia, nitrite and nitrate levels were daily checked, by means of colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Fish density was kept below 1 g body weight L^{-1} (i.e. 5 fish in each 50 L replicate tank) in order to avoid physiological stress related to high animal density. Specimens were initially acclimated to laboratory conditions for 30 days, being fed with CTR feed (2% of average body weight, BW) and kept under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (T ^{0}C) = 19.0 ± 0.5 °C; iii) pH = 8.00 ± 0.10 units; iv) salinity = 35 ± 1‰; and v) photoperiod = 12 L:12D (12 h light:12 h dark). Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Seawater total alkalinity was also measured in every tank on a weekly basis, following the protocol previously described elsewhere (Sarazin et al., 1999) and the combination of total

Table 1
Fish weight (g), total length (cm), VFX concentrations and net accumulation rates (NAR) in plasma of specimens collected in each treatment (trials I and II; day 28. In each column, different letters indicate significant differences between treatments ($p < 0.05$). Abbreviations: LOD: method's limit of detection; nd – not determined; Acid – simulated acidification (i.e. pCO_2 ~1500 μatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. $T = 24$ °C); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

	Weight (g)	Total length (cm)	Plasma VFX concentration (μg L^{-1})	NAR (μg L^{-1} day^{-1})
Day 0 (all)	2.6 ± 0.8^{ab}	6.8 ± 0.6^{abc}	nd	–
Control	2.3 ± 0.7^{b}	6.1 ± 0.7^{bc}	<LOD	–
VFX-water	2.2 ± 0.5^{b}	6.3 ± 0.4^{bc}	1292.0 ± 79.9^{a}	45.88 ± 1.72^{a}
VFX-feed	2.2 ± 0.5^{b}	6.0 ± 0.6^{c}	13.5 ± 1.4^{c}	0.48 ± 0.03^{d}
Acid	2.9 ± 0.8^{ab}	6.9 ± 0.6^{abc}	nd	–
Acid + VFX-feed	2.4 ± 0.4^{b}	6.5 ± 0.4^{bc}	24.8 ± 8.5^{bc}	0.91 ± 0.18^{c}
Warm	4.9 ± 1.1^{a}	7.8 ± 0.8^{a}	nd	–
Warm + VFX-feed	4.7 ± 1.6^{a}	7.8 ± 0.8^{a}	34.9 ± 20.6^{bc}	1.18 ± 0.44^{bc}
Acid + warm	4.8 ± 1.5^{a}	7.9 ± 0.5^{a}	nd	–
Acid + warm + VFX-feed	5.0 ± 1.8^{a}	8.0 ± 1.2^{ab}	40.6 ± 11.7^{b}	1.41 ± 0.25^{b}

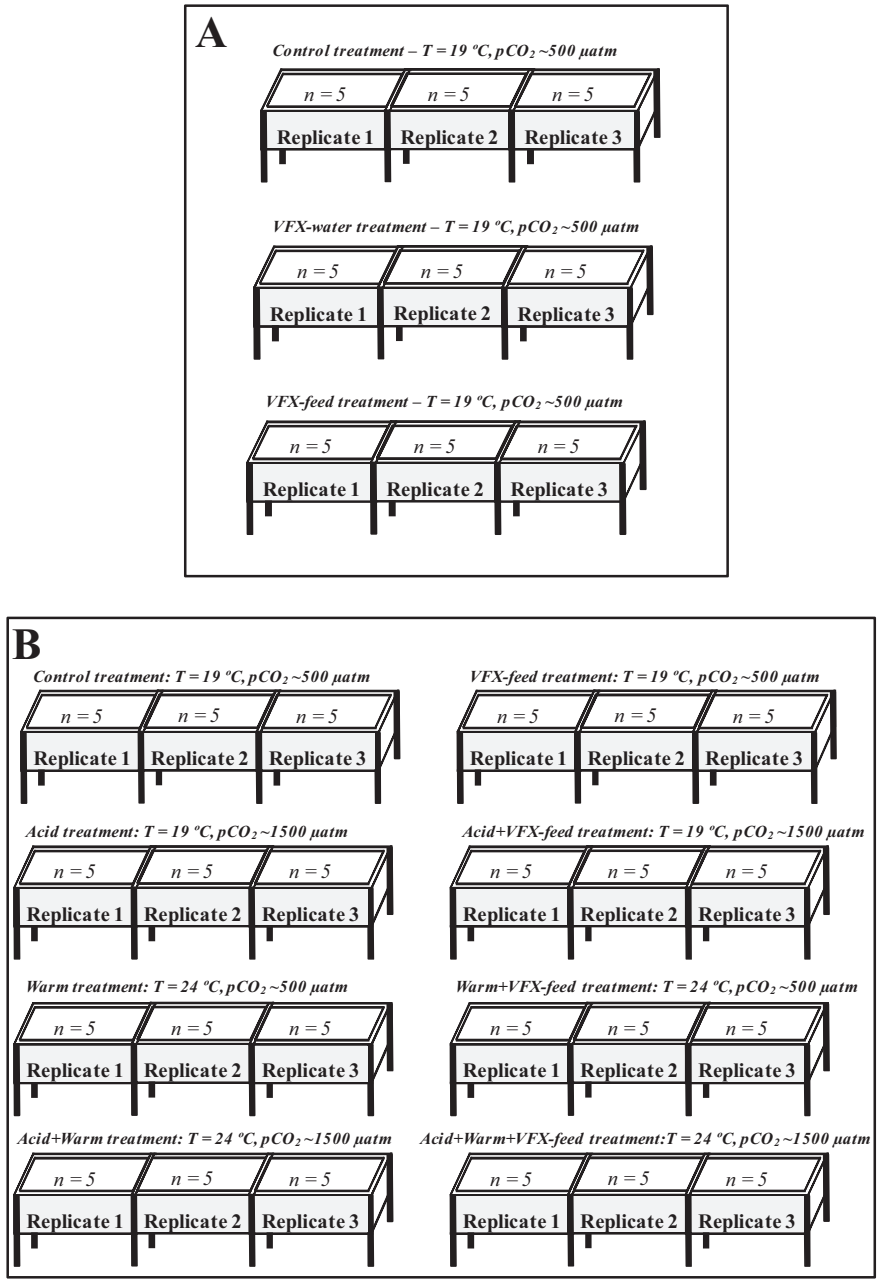

Fig. 1. Experimental design of trials I - Seawater *versus* dietary exposure (A); and II - Simulation of climate change effects and VFX exposure via enriched feed (B). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in Supplementary materials Table S.2).

2.3. Exposure to VFX

2.3.1. Trial I: Seawater versus dietary exposure

Three treatments were carried out ($n = 5$ animals per replicate tank, i.e. 15 animals per treatment; Fig. 1A), simulating the average seawater temperature and pH currently used in juvenile meagre rearing in the South Europe, i.e. reference temperature and pH conditions = 19 °C and 8.0 pH units: i) Control treatment, i.e. fish daily fed with CTR feed (2% BW); ii) VFX-water treatment, i.e. fish daily fed (2% BW) with CTR feed, and seawater daily spiked with a VFX stock solution (nominal concentration = 20 µg L^{-1} in the tank); iii) VFX-feed treatment, i.e. daily fish fed (2% BW) with VFX-enriched feed (nominal concentration = 160 µg kg^{-1} dw). Seawater abiotic parameters were daily checked and adjusted to adequate levels whenever needed, as described above. No mortality was observed during the 28 days of trial. By the end of exposure, behavioural tests were conducted (see Section 2.5) in ten animals randomly selected out of the three replicate tanks composing each treatment. Afterwards, fish were removed from the test tanks and euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 to 1 L of seawater) for 10 min. Euthanized fish were measured (total length and weight; Table 1), and blood was collected by puncture of the caudal vein and centrifuged (4 °C, 15 min, 10,000 *g*). Plasma samples were collected from the 10 fish for each treatment (3 tanks per treatment), pooled in two composite samples ($n = 2$) and kept at −80 °C until further analyses.

2.3.2. Trial II: Simulation of warming and acidification

Due to experimental limitations, only one exposure pathway was selected to investigate the link between VFX exposure and climate change. Thus, exposure via VFX-enriched feed was selected for this purpose, because: a) contaminant exposure through dietary sources (i.e. trophic transfer of contaminants) currently represents a research gap in ecotoxicological studies; b) dietary exposure is thought to significantly contribute to high contaminant bioaccumulation in animal tissues, sometimes leading to more notorious toxicological effects than those promoted by contaminant exposure through inhalation, depending on the chemical behaviour of the target contaminant (e.g. Arnot and Gobas, 2004; Brooks, 2014).

One week before initiating VFX exposure, seawater temperature and pCO_2 were slowly adjusted (+1 °C and −0.1 pH unit per day), until reaching 24 °C and ~1500 μatm pCO_2 (equivalent to pH = 7.6 units) in tanks simulating climate change conditions (i.e. treatments Acid, Warm, Acid+Warm, Acid+VFX-feed, Warm+VFX-feed and Acid +Warm+VFX-feed; Fig. 1B; see also Section 2.3.), according to the projections of the Intergovernmental Panel for Climate Change (scenario RPC8.5; IPCC, 2014). It is worth noting that the high pCO_2 levels used here (~1500 μatm) are beyond the worst-case IPCC scenarios for the end of the century (RCP8.5) (IPCC, 2014), but still within the intervals of future CO_2 amplification scenarios described by McNeil and Sasse (2016).

Eight treatments were carried out ($n = 5$ animals per replicate tank of treatment, i.e. a total of 15 animals per treatment; Fig. 1B), simulating the reference temperature (i.e. 19 °C) and pCO_2 (~500 μatm; 8.0 pH units) conditions, as well as the projected seawater warming (ΔT°C = +5 °C) and acidification (Δ pCO_2 ~1000 μatm; equivalent to ΔpH = −0.4 units), using a full cross-factorial design: i) Control treatment, i.e. fish daily fed with CTR feed (2% BW) and exposed to reference temperature and pH conditions; ii) Acid treatment, i.e. fish daily fed with CTR feed (2% BW) and exposed to acidification (1500 μatm pCO_2, equivalent to pH = 7.6 units); iii) Warm treatment, i.e. fish daily fed with CTR feed (2% BW) and exposed to warming (24 °C); iv) Acid + Warm, i.e. fish daily fed with CTR feed (2% BW) and exposed to warming and acidification (24 °C and ~1500 μatm pCO_2); v) VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% BW) and exposed to reference temperature and pH conditions; vi) Acid+VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% BW) and exposed to acidification (1500 μatm pCO_2); vii) Warm + VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% BW) and exposed to warming (24 °C); viii) Acid + Warm + VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% BW) and exposed to acidification and warming (24 °C and ~1500 μatm pCO_2). Seawater abiotic parameters were daily checked and adjusted to adequate levels whenever needed, as described above. No mortality was observed during the 28 days of trial. By the end of exposure, behavioural tests were conducted (see Section 2.5) in ten animals randomly selected out of the three replicates composing each treatment. Afterwards, fish were removed from the test tanks and euthanized with MS222, as previously described. Biometric data were registered (Table 1), and plasma samples were collected from the 10 fish (as described for Trial I, Section 2.3.1) for each treatment (3 tanks per treatment), pooled in two composite samples ($n = 2$) and kept at −80 °C until further analyses.

2.4. Venlafaxine determination

Seawater samples were collected from each tank (treatment) in both trials, at days 0, 14 and 28 of the experiment, filtered by PVDF syringe filters 0.22 μm (Merck Millipore) and VFX was quantified by direct injection in UPLC-QqLIT according to the methodology described by Gros et al. (2012). Monitoring VFX concentrations in water along the trials allowed to: a) Trial I - assure a steady VFX concentration throughout the experiment in tanks/treatments simulating VFX exposure via water; b) Trials I and II - assure that no external contamination (apart from the intended dietary exposure) was also taking place in tanks/treatments simulating VFX exposure via feed. VFX concentrations were determined in composite samples of fish plasma (day 28; Table 1) to establish possible relationships between fish behaviour and VFX bioaccumulation in the different treatments. For the analysis of VFX in fish plasma, 50 μL of plasma were mixed with 50 μL of methanol and centrifuged (5000 rpm, 10 min, 4 °C). Then, 60 μL of supernatant were transferred to an insert and 0.6 μL of a 1 ng μL^{-1} VFX-d6 standard solution was added before the analysis by UPLC-QqLIT using the methodology described by Gros et al. (2012). Finally, VFX levels in feed (both, control and VFX-enriched) were determined using an extraction methodology adapted from Jakimska et al. (2013) and further quantified by UPLC-QqLIT by Gros et al. (2012). Data generated was considered satisfactory thanks to the good precision and accuracy of the analytical methodologies previously optimized and validated (Gros et al., 2012; Jakimska et al., 2013), and detailed information on the validation parameters is given in Supplementary materials, Table S3. Venlafaxine net accumulation rates for each treatment (NAR; μg L^{-1} day^{-1}) were determined assuming that fish were exposed to steady conditions (i.e. continuous contaminant exposure, as well as seawater abiotic parameters) and using the following equation (Santana et al., 2017):

$$NAR_t = \frac{([VFX_{t28}] - [VFX_{t0}])}{t}$$

where, $[VFX]_{to}$ is the average VFX concentration in fish plasma before exposure (day 0) and $[VFX]_{t28}$ is the average VFX concentration after 28 days of exposure.

2.5. Behaviour assessment

The first two behaviour tests were conducted according to the Novel Tank assay (to test for anxiety; Test 1) previously described by Egan et al. (2009) and the Shoaling assay (to test social behaviour; Test 2) first described by Moretz et al. (2007), with some modifications (Reyhanian et al., 2011). Briefly, glass test tanks (50 × 26 × 26 cm each) filled with about 20 L of seawater were set up so that Tests 1 and 2 could be performed one after the other, in the same run (the set up for each test can be consulted in Fig. S1 of the *Supplementary Materials*). An isolated zone (8 × 26 × 26 cm), in the right end of each tank, was created using a transparent acrylic plate, to trap and separate a shoal of 5 fish from the testing area. Visual contact beforehand between the test fish and the shoal was avoided by placing a second acrylic plate covered with a black plastic sheet, next to the first plate. Then, the test tanks were horizontally divided, with gridlines marked in the outer part of the tanks, in order to define the bottom (B) and top (T) halves, and vertically divided defining: i) 2 zones for Test 1, i.e. left (L) and right (R) halves; ii) 3 zones for Test 2, i.e. close to the shoal (in_shoal), far from shoal (out_shoal-I) and very far from shoal (out_shoal-II). Before initiating the behaviour assessment, seawater temperature and pH were adjusted according to the experimental conditions set in each treatment (i.e. 19 °C or 24 °C, and pCO_2 = ~500 or ~1500 μatm equivalent to 8.0 pH units and 7.6 pH units, respectively), and fish were not fed 12 h prior to the tests. Behaviour tests were performed by direct observation, using in each test the same team of observers, in order to avoid inter-observer variability. Furthermore, to avoid the potential observer bias, all behaviour tests were performed in a blind way, i.e. no information was provided to the observers regarding the experimental groups that were being tested. In all tests, three test tanks were run at the same time, and behavioural observations were carried out between 8.00 and 14.00 am, to minimize data variability due to metabolic fluctuations (e.g. cortisol cycle) that normally occur in fish species along the day.

For the Novel Tank assay (Test 1; $n = 10$), the test fish was gently introduced in the test tank (by netting; 3 s of maximum time outside water), and the counting was initiated as soon as the fish reached the

bottom area of the tank. Then, the time spent before crossing, for the first time, the gridline into the top area of the tank (T) was registered, as well as the total time spent in T and the number of vertical grid transitions (from B to T, and from T to B), during 5 min of observation. Fish swimming activity was also evaluated in parallel (counts initiated 30 s after introduction in the test tank), by counting the total number of transitions gridline (horizontal, i.e. from L to R and R to L; and vertical, i.e. from B to T and T to B) during 1 min. After the 5 min time period of Test 1, the Shoaling assay (Test 2; $n = 10$) was immediately initiated by removing the black acrylic plate (leaving only the transparent one) and, thus, allowing the test fish to visualise the shoal. Then, the time spent before performing, for the first time, a transition towards the shoal (i.e. time to visualise the shoal for the first time) was registered, as well as the total time spent close to the shoal (in_shoal), and the number of gridline transitions towards or away from the shoal (i.e. from out_shoal-I or -II to in_shoal, and vice-versa; out_shoal-I counted as one gridline transitions and outshoal-II counted as two gridline transitions). Test 2 was concluded after a 5 min time period of observation. Fish that exhibited total immobility (i.e. did not show any swimming activity) during the 5 min of tests 1 and 2 were excluded from data analysis (i.e. only three cases: 1 fish from Control treatment, 1 fish from Acid + Warm treatment and 1 fish from Warm + VFX-feed treatment), as they were considered to be in an extreme (unusual) state of physiological stress, which could unlikely be exclusively attributed to the experimental conditions (i.e. VFX exposure, warming and acidification), based on the overall behaviour of the tested group.

Finally, the test fish previously used in Tests 1 and 2 was quickly and gently transferred (by netting; 3 s of maximum time outside water) to another test tank in which the Lateralization Assay (Test 3; $n = 10$) was carried out (see Supplementary materials, Fig. S1), and allowed to acclimate for a period of 5 min (seawater temperature and pH adjusted according to the experimental conditions in each treatment). Test 3 was, then, performed according to the detour test previously described by Bisazza et al. (1998), briefly consisting of a two-way T-maze with a central runway and a movable wall at the end. The test fish was placed in one end of the tank (i.e. the starting point) and compelled to swim forward (by approaching with a scoop, simulating a potential threat). Once it reached the wall, the fish had to choose which way to turn, i.e. left (L) or right (R), to escape. Ten consecutive runs per test fish were carried out, and the turning side was visually scored. To minimize possible irregularities in the test tank, both ends of the T-maze were alternatively used during the 10 runs. The relative lateralization index (L_R) was calculated for each fish according to Bisazza et al. (1998):

$$L_R = [(\text{turns to the right} - \text{turns to the left})/(\text{turns to the right} + \text{turn to the left})] \times 100$$

with values close to 100 representing fish that turned right in all 10 runs, −100 representing fish that chose left instead in all 10 runs, and values near zero representing fish that equally preferred left and right. Fish absolute lateralization (i.e. the absolute L_R value for each fish; L_A) was also calculated, with values close to 0 indicating an equal preference for left and right, and values close to 100 indicating a preference for left or right in a total of 10 runs.

2.6. Statistical analysis

To determine significant differences among treatments in VFX plasma concentrations (and NAR), after checking that data complied with assumptions of normality (Kolmogorov–Smirnov's test) and homogeneity of variances (Levene's test), the analysis of variance ANOVA was carried out. Pearson correlation coefficients (r) between biometric data and VFX concentrations in fish plasma from each treatment were also calculated. For behavioural data, treatment effects were studied using Generalized Linear Mixed Models (GLMM), with tank replicate as random effect. A Gaussian distribution was used to analyse continuous data (i.e. latency to the top, latency to move towards the shoal and lateralisation), whereas a binomial distribution (or negative binomial when over-dispersion was observed) for proportions was used (percentage of time spent in the top, percentage of time spent within the shoal and percentage of transitions towards the shoal). Moreover, negative binomial distribution was also used for total number of transitions (i.e. fish activity) to account for over-dispersion. Selection for best model was made using Akaike Information Criterion (AIC), and the summary of GLMM results is presented in Supplementary Materials, Table S4. Model assumptions, namely independence and absence of residual patterns, were verified by plotting residuals against fitted values and each covariate in the model. The post-hoc Tukey test was also carried out for multiple comparisons (see Figs. 2–4 and Table 2). Statistical analysis was performed in R (R Core Team, 2017) and data exploration and model validation used the R library from Highland Statistics (Zuur et al., 2009). Statistical analyses were performed at a significance level of 0.05.

3. Results

3.1. Biometric parameters and VFX concentrations

Matching the nominal concentration selected for the contaminated feed, VFX concentration in VFX-enriched feed was around 161.7 ± 17.1 μg kg^{-1} dw, whereas VFX was not detected in CTR feed confirming that no external contamination occurred during feed preparation. In seawater samples VFX was only found in detectable levels in VFX-water treatment (day 0: <detection limit, i.e. <0.15 μg L^{-1}; day 14: 20.9 ± 1.8 μg L^{-1}; day 28: 19.2 ± 1.6 μg L^{-1}), thus confirming that: i) in Trial I, VFX concentration was maintained at around 20 μg L^{-1} in VFX-water treatment throughout the 28 days of exposure; ii) no contamination occurred, apart from the intended contamination of the feed, in VFX-feed, Acid + VFX-feed, Warm + VFX-feed and Acid + Warm + VFX-feed treatments (Trial II).

Biometric parameters (i.e. weight, W, and total length, TL) of fish collected from each treatment in Trials I and II, as well as VFX concentrations in fish plasma are shown in Table 1. In Trial I, W and TL did not significantly vary among treatments ($p > 0.05$), whereas in Trial II, in overall, fish exposed to warmer temperature exhibited significantly higher W and TL ($p < 0.05$), regardless of seawater pH and VFX absence/presence. The maximum values were observed in the Acid + Warm + VFX-feed treatment (weight = 5.0 ± 1.8 g; total length = 8.0 ± 1.2 cm; Table 1).

No detectable levels of VFX were observed in plasma of control specimens, confirming that there was no other sources of external contamination apart from intended contamination of water (VFX-water treatment; Trial I) or feed (all VFX-feed treatments; Trials I and II). In Trial I, VFX plasma levels were much higher in fish exposed via water compared to fish exposed via feed (~50× higher in VFX-water; $NAR_{VFX\text{-}water} = 46\ \mu g\ L^{-1}\ day^{-1}$ against $NAR_{VFX\text{-}feed} = 0.5\ \mu g\ L^{-1}\ day^{-1}$; Table 1). In Trial II, VFX concentrations in plasma from fish under the control seawater temperature and pH conditions were significantly lower than those in fish exposed to warmer temperature and lower pH simultaneously (i.e. 13.5 ± 1.4 μg L^{-1} against 40.6 ± 11.7 μg L^{-1} in VFX-feed and Acid + Warm + VFX-feed, respectively; $p < 0.05$), but not when fish were exposed to either of the two stressors acting alone (i.e. 24.8 ± 8.5 μg L^{-1} and 34.9 ± 20.6 μg L^{-1} in Acid + VFX-feed and Warm + VFX-feed, respectively; Table 1). Significantly higher NARs were determined in fish exposed to warming and acidification, acting alone or in combination, compared to those exposed to the control temperature and pH (i.e. VFX-feed; $p < 0.05$; Table 1).

Significant positive correlations were found between W or TL and VFX concentrations, regardless of exposure pathway (W: $r = 0.78$ and $r = 0.70$ for VFX water and feed exposure treatments, respectively; TL: $r = 0.66$ and $r = 0.75$ for VFX water and feed exposure treatments, respectively; $p < 0.01$).

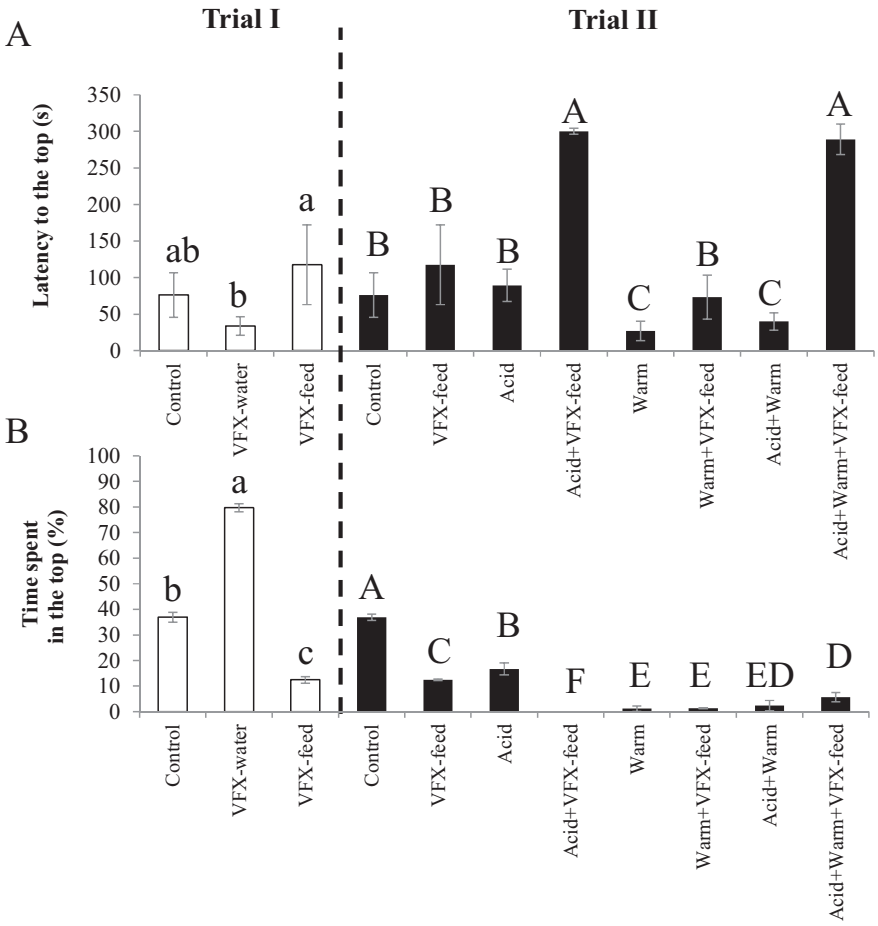

Fig. 2. Latency to reach the top area of the tank, for the first time (2A) and percentage of time spent in this area (2B), during 5 min of the test ($n = 10$; mean ± standard deviation). Different lower case letters indicate significant differences between treatments in Trial I, whereas upper case letter indicate significant differences between treatments in Trial II ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 μatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

3.2. Behavioural assays

In Trial I, no significant differences between non-contaminated and contaminated fish were observed in the time spent before initiating the exploration of the upper part of the tank (T; Fig. 2A). On the other hand, VFX exposure significantly affected the total time spent within T, regardless of exposure pathway, increasing in fish exposed via water and decreasing in those exposed via feed ($p < 0.05$; Fig. 2B). Activity levels were significantly decreased in VFX contaminated fish (30 ± 12 grid movements in control treatment, against 11 ± 5 and 7 ± 3 in VFX-water and VFX-feed, respectively; $p < 0.05$; Table 2).

In the test of social behaviour (shoaling test), despite fish exposed to VFX via feed took less time to perform, for the first time, a transition towards the shoal (Fig. 3A), the percentage of transitions made towards the shoal was not significantly affect by VFX (both exposure routes; Fig. 3B)·On the other hand, the total time spent within the shoal was drastically decreased in fish exposed to VFX via water ($p < 0.05$), and even further decreased with VFX exposure via feed ($p < 0.05$; Fig. 3C). Overall, control fish exhibited a preference to turn leftwards (L_R; Fig. 4A). This pattern was maintained in fish exposed to VFX via water, but not in fish from VFX-feed treatment, in which L_R and L_A values closer to zero were observed ($p < 0.05$, for L_R; Fig. 4).

In Trial II, significantly different behavioural patterns were observed in fish exposed to increased temperature and high pCO_2, when acting alone or in combination with VFX exposure (Figs. 2–4; Table 2). Starting with the introduction to a novel environment, acidification combined with VFX exposure significantly increased the time spent before initiating the exploration of the upper areas of the tank (T), regardless of temperature (i.e. treatments Acid + VFX-feed and Acid + Warm + VFX-feed ($p < 0.001$; Fig. 2A). Moreover, fish exposed to VFX, acidification and/or warming tended to spend less time on the upper area of the tank compared to those from Control treatment ($p < 0.05$; Fig. 2B). In terms of overall fish activity, acidification by itself increased the total number of grid transitions in comparison to all the other treatments, whereas in Acid + VFX-feed treatment the number of transitions drastically decreased and no transitions to the top were observed ($p < 0.001$; Table 2). During the shoaling test, in both non-contaminated and VFX-enriched fish, warming in combination with acidification increased the time spent before fish performed the first transition towards the the shoal ($p < 0.05$; Fig. 3A), but not when these two stressors acted independently (regardless of VFX exposure). Despite no significant differences were observed among treatments in the total number of transitions towards the shoal, fish exposed to VFX and/or acidification (i.e. VFX-feed, Acid, Acid + VFX-feed, Acid + Warm and Acid + Warm + VFX-feed) spent significantly less time spent within the shoal formation compared to fish from Control, Warm and Warm + VFX-feed treatments ($p < 0.05$; Fig. 3C). Furthermore, in general, fish exposed to acidification and/or warming (with and without VFX)tended to stay within the shoal for a longer period of time than contaminated fish subjected to reference temperature and pCO_2 conditions (i.e. treatment VFX-feed; $p < 0.05$; Fig. 3C). VFX feed exposure, acidification and/or warming affected fish lateralization, with fish exposed to acidification evidencing a preference to turn rightwards, as opposed to the control, VFX-feed and Warm+VFX-feed

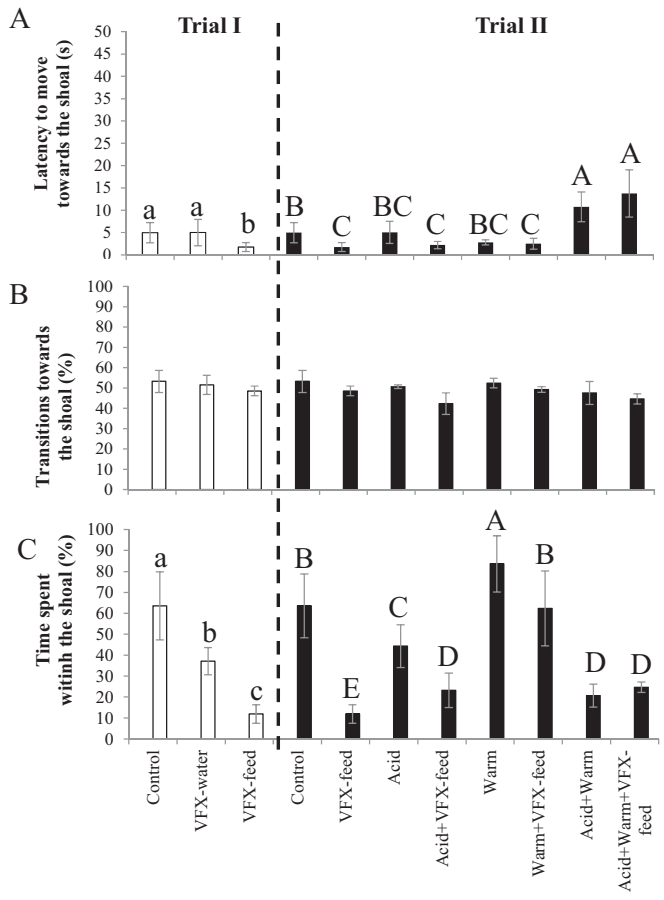

Fig. 3. Time spent until fish visualized the shoal for the first time (3A), percentage of transitions towards the shoal (3B), and percentage of time spent in this area (3C) during the 5 min of shoaling test ($n = 10$; mean ± standard deviation). Different lower case letters indicate significant differences between treatments in Trial I, whereas upper case letter indicate significant differences between treatments in Trial II ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 μatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

treatments in which a turning preference to the left was observed ($p < 0.01$; Fig. 4A). Moreover, an overall loss of preference was observed compared to non-contaminated fish (i.e. lower L_A, with exception of Warm + VFX-feed treatment), particularly in fish exposed to combined acidification and VFX (i.e. Acid + VFX-feed; $L_A > 20$; $p < 0.01$), as well as when the three stressors were combined (i.e. Acid + Warm + VFX-feed; L_A ~10; $p < 0.01$; Fig. 4B).

4. Discussion

4.1. Effects of exposure route, temperature and pCO_2 on VFX bioaccumulation

The present results confirmed that VFX can be accumulated, not only from water, but also from diet (and, therefore, biomagnified along the food chain), thus, further emphasizing the ecological hazards this compound can pose to marine ecosystems. Yet, the lower VFX plasma concentration and NAR in fish exposed via feed suggest that diet may indeed play a minor role on the uptake of these ionizable weak base pharmaceuticals, compared to other routes of exposure (such as inhalation), as demonstrated in a recent laboratory study using several aquatic species from different trophic levels exposed to sertraline and fluoxetine (also anti-depressants; Boström et al., 2017). In fish exposed to VFX via feed, increased temperature and pCO_2 levels enhanced VFX bioaccumulation, thus, evidencing the need to consider the potential interactions with abiotic variables when assessing the ecotoxicological implications of pollutants. Such increase in VFX bioaccumulation is most likely related to the metabolic changes induced by altered temperature and pCO_2 (e.g. Rosa et al., 2016; Sampaio et al., 2016, 2018), as well as possible tissue damages, which can then facilitate contaminant penetration into cells (Freitas et al., 2016; Sampaio et al., 2016, 2018; Shi et al., 2016; Velez et al., 2016).

The discrepancy of VFX plasma concentrations and NAR between the two exposure routes may, at a first glance, point out to the favoring of VFX bioaccumulation when exposure occurs via water, a result that is consistent with VFX physical-chemical properties (e.g. log Kow between 2.74 and 3.30; Aryal et al., 2012). However, it should be noted that the selected VFX concentrations for water and feed are not comparable (and compound bioaccumulation patterns may be dose-dependent) nor the present experimental design allowed to investigate some parameters (e.g. respiration and ingestion rates, compound bioavailability) which are crucial to deeply assess compound toxicokinetics (i.e. such knowledge was outside the scope of this work). Particularly in what concerns VFX exposure via water, the ratio between VFX concentrations in fish plasma and seawater (i.e. the bioconcentration factor, BCF) obtained in the present study (BFC = 64.6 ± 0.4) was much higher than the values previously reported for fish plasma (i.e. BFC = 8; Grabicova et al. (2014) and brain (BCF around 10; Lajeunesse et al., 2011; Grabicova et al., 2014), suggesting that VFX bioaccumulation may be dose dependent. As for the relationship between VFX plasma concentration and fish morphometry, as observed for other chemical contaminants such as MeHg (e.g. Dijkstra et al., 2013; Maulvault et al., 2016), results showed that VFX bioaccumulation in fish plasma was directly linked (i.e. correlated) to animal growth, regardless of the exposure pathway. Furthermore, because growth and contaminant metabolization/excretion are also largely influenced by abiotic conditions, changes in seawater temperature and pH can lead to increased contaminant bioaccumulation (e.g. Dijkstra et al., 2013; Maulvault et al., 2016; Sampaio et al., 2016), as observed in Trial II, particularly, when warmer temperatures and high pCO_2 levels were combined.

4.2. Differential effects of VFX exposure route on fish behaviour

Both water and feed exposure triggered significant behavioural alterations, though to different extents and in different directions, and such differences are likely related to the different VFX levels reached in fish plasma in these treatments. Despite the lower concentrations detected in fish plasma, VFX exposure via diet (VFX-feed) significantly affected fish behavior and response to stress, when compared to non-contaminated fish or even to fish exposed to VFX via water (Control and VFX-water). This shows the great ability for this pharmaceutical to easily cross the brain blood barrier (i.e. BCF ~10 in fish brain; Lajeunesse et al., 2011; Grabicova et al., 2014) and promote severe behavioural alterations, even at lower VFX plasma concentrations (Bisesi Jr. et al., 2014), as those elicited by VFX exposure via feed.

Differences between the two exposure routes (i.e. water and feed) were particularly evident in terms of fish exploratory activity and social interactions, which are two ecologically determining factors (Reyhanian et al., 2011; Stewart et al., 2012). Decreased exploratory activity along with increased erratic movements, latency to reach top areas and freezing have been typically associated with increased plasma levels of stress hormones such as cortisol and, consequently, to anxiety in fish (Wibe et al., 2002; Egan et al., 2009). Similarly to the effects induced in both humans and rodents (Katzman, 2004; Sprowles et al., 2017), chronic or acute exposure to different antidepressants has shown to decrease fish anxiety (e.g. citalopram in *Danio rerio*, Sackerman et al., 2010; fluoxetine in *Pimephales promelas*, Margiotta-Casaluci et al., 2014; fluoxetine in *Pachygrapsus crassipes*, Hamilton et al., 2015). Yet, an interesting outcome of the present study was that, in the novel tank test, the anxiolytic effect of VFX was clearly verified in fish exposed to this antidepressant via water (significantly longer permanence in the top area of the tank,

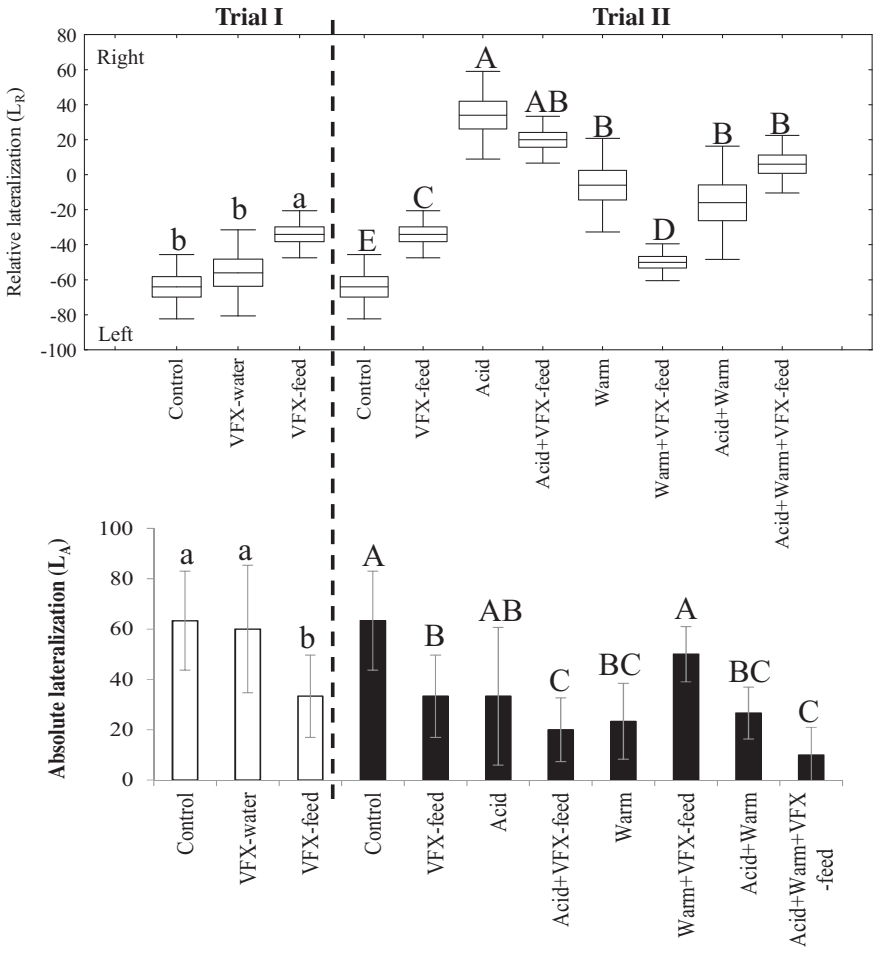

Fig. 4. Relative lateralization (4A; L_R; $n = 10$; box plots) and absolute lateralization (4B; L_A; $n = 10$; mean ± standard deviation) in *A. regius* after 28 days of exposure to VFX, warming and acidification. Different lower case letters indicate significant differences between treatments in Trial I, whereas upper case letter indicate significant differences between treatments in Trial II ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 μatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

despite the lower number of transitions compared to CTR fish), but not in fish exposed to VFX via feed. Such differences between the two contaminated treatments could be related to dose-dependent action of VFX (which is in line with the higher VFX concentrations in plasma of fish exposed via water) and/or to distinct bioavailability of this compound according to the exposure route (Sanchez and Meier, 1997; Brooks, 2014; Gray and Hughes, 2015).

Although the apparent state of decreased anxiety in fish exposed to VFX via water (i.e. increased exploratory behaviour) may, at a first glance, come as somewhat positive (e.g. resulting in increased opportunities for feeding, reproduction and territory establishment in the wild), it can also translate into increased risk of predation, which is not beneficial from the ecological point of view. Hence, an increased bottom-dwelling behaviour can be also looked at as an anti-predatory strategy (Maximino et al., 2012). Noteworthy, the increased preference for the bottom of fish exposed to VFX via feed may also be related to a combination of locomotor (increased sedation) and motivational (anxiolytic-anxiogenic) effects induced by VFX dietary exposure (Maximino et al., 2012; Rosemberg et al., 2010), as it can be corroborated by the lower number of transitions compared to control fish.

Apart from playing a key role as an energy-saving mechanism during swimming, foraging and mating, close shoaling also represents an important anti-predatory strategy, increasing the chances of survival in face of danger, at both the individual and group levels (Pitcher and Parrish, 1993). Here, VFX exposure via water or feed decreased fish tendency to stay within the shoal formation, possibly as a result of lower fish anxiety, which can likely constitute an ecological drawback in the wild (Maximino et al., 2012). A similar trend was also described in study on *D. rerio* exposed to different contaminants with anxiolytic properties (i.e. clonazepam, bromazepam, diazepam, buspirone, propranolol and ethanol; Gebauer et al., 2011). Despite previously evidencing signs of increased anxiety compared to Control and VFX-water treatments, the 5 min spent during the first test (novel tank), which worked as an acclimation period before initiating the second test (shoaling), might have contributed to progressively drive fish from VFX-feed treatment into a lower stage of anxiety (i.e. less time spent before performing the first transition towards the shoal compared to

Table 2
Number of transitions made to each of 4 halves of the test tank during 1 min of activity test ($n = 10$; mean ± standard deviation). In each column, different lower case letters indicate significant differences between treatments ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 μatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

	Total grid movements
Control	30 ± 12^{bc}
VFX-water	11 ± 5^{d}
VFX-feed	7 ± 3^{de}
Acid	80 ± 10^{a}
Acid + VFX-feed	2 ± 2^{e}
Warm	22 ± 4^{c}
Warm + VFX-feed	5 ± 2^{de}
Acid + warm	45 ± 9^{b}
Acid + warm + VFX-feed	30 ± 7^{bc}

Control and VFX-water, as well as similar number of transitions towards the shoal in these three treatments).

The fact that lateralization (due to brain asymmetry) prevails within the animal kingdom suggests that this feature may represent a selective advantage over bilateral control of the cognitive functions (Rogers, 2002; Bisazza and Dadda, 2005). Despite the lack of statistical significance between Control and VFX-water treatments, which could be related to several factors (e.g. individual temperamental characteristics, drug sensitivity, and bias effects specifically associated to the chosen lateralization test, i.e. detour test), the present data suggests that side preference was decreased by VFX exposure via feed. Furthermore, as described by other authors (Bisazza and Dadda, 2005; Bisazza et al., 2000; Bisazza and Brown, 2011), the impairment of side preference promoted by VFX exposure via feed can also be linked to diminished social interactions, thus, matching the diminished time spent within the shoal in VFX-feed treatment, regardless of the number of transitions made on that direction.

4.3. Combined effects of VFX exposure, warming and acidification on fish behaviour

Warming and acidification, alone or combined, significantly enhanced or attenuated the effects of VFX exposure on fish stress response, social skills and lateralization. Elevated pCO_2 levels are known not only to increase animal anxiety and boldness, but also to impair lateralization and olfaction, most likely due to the disruption of the ionic balance in proton-based neurotransmitter receptors, such as $GABA_A$ (e.g. Nilsson et al., 2012; Hamilton et al., 2014; Munday et al., 2014; Sampaio et al., 2016; Lai et al., 2015). For instance, following a light/dark test (scototaxis), Hamilton et al. (2014) reported increased anxiety in juvenile Californian rockfish (*Sebastes diploproa*) exposed to acidification, compared to specimens exposed to normal conditions. Another study using *Atherina presbyter* larvae, also reported decreased shoal cohesion after 7 days of exposure to high pCO_2, as well as individual loss of lateralization (Lopes et al., 2016). Here, in Trial II, significant behavioural changes were observed in fish exposed to acidification, particularly in terms of fish anxiety (less time spent in the top area), activity (increased number of transitions), and lateralization (reversed side preference). Such behavioural effects were further enhanced by VFX exposure via feed, which translated into a substantial decrease of fish swimming activity, exploration and time spent in shoal formation, as well as the loss of side preference that was clearly observed in fish exposed to acidification alone (towards the right). This constitutes an interesting outcome, since VFX acts as an anxiolytic (in humans) and, therefore, a counteraction of the anxiety induced by acidification would be expected instead. Such results further suggest that another mechanism, apart from the altered Cl^- flow through $GABA_A$ receptors one induced by acidification alone, may be involved when VFX and acidification are combined (Nilsson et al., 2012; Hamilton et al., 2014), thus, calling for the need to further explore and understand the neurophysiological mechanisms involved when multiple stressors (such as VFX and acidification) interact.

As for the effects of warming, so far, the majority of studies is primarily focused on metabolic changes and physiological stress induced by thermal stress (e.g. Nilsson et al., 2012; Rosa et al., 2016), whereas little is known regarding its impacts on behavioural cues, such as shoaling and lateralization. Warmer temperatures have been often associated to increased activity and boldness (e.g. Forsatkar et al., 2016). Yet, this was not observed in the present study, as fish exposed to warming exhibited similar swimming activity and spent less time exploring the top area of the tank than Control fish. As for lateralization, a trend similar to the one observed in the present study was also reported by Domenici et al. (2014) in Ward's damselfish (*Pomacentrus wardi*), with warmer temperatures attenuating the bias observed in control treatments, or even reversing the effects promoted by acidification.

The combination of VFX exposure, warming and acidification seemed to have elicited even more drastic behavioural changes (i.e. increased activity, as well as increased latency for the top area and towards the shoal) compared to each of these stressors acting alone, a result that is aligned with the higher VFX plasma concentration that was also observed in this treatment, thus pointing out to the great ecological impacts involved when the three stressors occur simultaneously. Similarly, in our previous study using juveline flatfish *Solea senegalensis*, distinct behavioural patterns were also observed when three stressors (MeHg exposure, warming and acidification) acted alone or combined, with increased temperatures reversing the effects of acidification in terms of fish boldness and decision making in non-contaminated fish, whereas such reversion did not occur when MeHg exposure was also added to the equation (Sampaio et al., 2016). To sum up, the present study constitutes a proof of concept that warming, acidification and contaminant exposure can have differential and interactive effects on fish behaviour. Yet, it should be noted that the present findings are limited to the selected levels for VFX exposure and seawater temperature and pCO_2 altered conditions (i.e. only one exposure level was tested for each stressor, given the complexity of the experimental design already as it was) and, therefore, bioaccumulation/behavioural patterns may differ when fish are exposed to a lower or higher severity degree of these stressors.

5. Conclusions

The present study showed that: i) the way, extent and direction in which VFX affects fish behavior is strongly related to exposure route and VFX concentration reached in fish plasma; ii) climate change-related stressors, particularly acidification, significantly affect fish behaviour, which can then translate into deleterious ecological impacts; and iii) such behavioural alterations can be further accentuated or reversed in some instances when these abiotic stressors interact with each other, or when chemical contamination occurs.

The present findings constitute a relevant proof of concept, not only reinforcing the suitability of fish species to assess the toxicokinetics and behavioural implications of SNRI antidepressants, like VFX, but also evidencing the deleterious ecological impacts of human pharmaceutical pollutants on marine vertebrates in tomorrow's ocean. This calls for a better understanding of the ecotoxicological impacts of these compounds on non-target marine vertebrate species, particularly focusing on their different modes of action and bioavailability. Furthermore, the distinct behavioural patterns observed when VFX contamination, high pCO_2 and warming acted alone or in combination also highlight the urgent need to consider multiple environmental stressors (exploring less pronounced to more severe exposure scenarios) in future behavioural ecology studies. Such studies are particularly important since both environmental pollution and climate change effects are expected to worsen in the coming years, and the ecological consequences associated to these stressors, as well as to their interactions with other stressors are still far from being completely understood.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63), and approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.04.015.

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